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A STUDY OF MURINE BONE MARROW CELLS CULTURED IN BIOREACTORS
WHICH CREATE AN ENVIRONMENT WHICH SIMULATED MICROGRAVITY

P-15

Final Report

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ABSTRACT

This study is a continuation of work undertaken at Johnson Space Center in the summer, 1989. That research indicated that mouse bone marrow cells could be grown in conditions of simulated microgravity. This environment was created in rotating bioreactor vessels, which were fabricated by the Biotechnology Instrument Laboratories of the Johnson Space Center.

On three attempts mouse bone marrow cells were grown successfully in the vessels. The cells reached a stage where the concentrations were doubling daily. Phenotypic analysis using a panel of monoclonal antibodies indicated that the cells were hematopoietic pluripotent stem cells. They did not show surface markers characteristic of differentiated myeloid, lymphoid or erythroid cells. Furthermore, they stained positively for an antibody which has been shown to be present on stem cells. These cells formed colonies when grown on soft agar. Morphologically, they appeared clonic. One unsuccessful attempt was made to reestablish the immune system in immunocompromised mice using these cells.

Since last summer, several unsuccessful attempts were made to duplicate these results. It was determined by electron microscopy that the cells successfully grown in 1989 contained virus particles. It has been suggested that these virally parasitized cells had been immortalized,

The work of this summer is a continuation of efforts to grow mouse bone marrow in these vessels. A number of variations of the protocol have been introduced. Certified pathogen free mice have been used in the repeat experiments. In some attempts the medium of last summer was used; in others Dexture Culture Medium containing Iscove's Medium supplemented with 20% horse serum and 10^{-6} M hydrocortisone. The Dexter medium proved to be unsuitable for growth in the vessels but was excellent for growth of static cultures at unit gravity.

Our efforts this summer were directed solely to repeating the work of last summer. We had planned many investigations if we succeeded in isolating the stem cells. We would attempt immortalization of the undifferentiated stem cell by transfection with oncogenic vector. We would induce selective differentiation in the stem cell line by growing it with known growth factors and immune response modulators. We were particularly interested in identifying any surface antigens unique to stem cells that would help in their characterization.

Another goal was to search for markers on stem cells that would distinguish them from stem cells committed to a particular lineage; these are the cells referred to as precursor cells. We believe that the stem cell is self-renewing while the precursor cell obeys the restrictions proposed by Leonard Hayflick (Hayflick, L., 1965).

If we obtained the undifferentiated hematopoietic stem cell we would study the pathways that would terminally convert it to myeloid, lymphoid, erythroid or other cell line. We would like to transfect it with a known gene and then convert it to a terminally identifiable cell.

INTRODUCTION

This research is a study of murine bone marrow cells cultured in simulated microgravity. It has been shown by several investigators that gravity is an environmental factor which affects growth and functionality of cells (Lorenzi, G., 1986). When flown on a space mission where they encountered zero gravity, human lymphocytes showed less than 3% of mitogenic activation to Concanavalin A as similar cells which had been cultured at unit gravity (Cogoli, 1985). It has been further demonstrated that microgravity depresses and hypergravity enhances cell proliferation rates. These effects are particularly strong in cells which are undergoing differentiation. The cellular proliferation rates of several different cell types were found to increase by 30% in hypergravity while the consumption per cell of glucose was lower than at unity gravity (Tschopp, 1983). A summary of the effects of spaceflight on single cell organisms, plant and mammalian cells has appeared in the literature. (Gmunder, F.K. and A. Cogoli, 1988). The effects of spaceflight on levels and activity of immune cells has been reported by Sonnenfeld, et al (1990).

We have undertaken this research encouraged by the work of these investigators and others who have demonstrated that gravity affects cell behavior. Our particular interest in bone marrow is associated with its importance in clinical medicine and several areas of basic research. It is important that the stem cell be isolated and characterized. The investigation of hematopoiesis is limited by the by the lack of a definitive in vitro assay for the most primitive hematopoietic stem cell. (Rowley, et al, 1987). No antigenic determinant has been found to date that is specific for mouse stem cells. The antigens that are positive for stem cells are not specific. (Van de Rijn, et al., 1989) It is estimated that only 0.02% of bone marrow cells are undifferentiated stem cells. It is postulated that these cells are the only ones with self-renewal potentialities. (Spangrude, et al., 1988).

Among the premises upon which this research is predicated are: (a) cell-cell contacts would be minimized in the bioreactors; (b) cell differentiations would be curtailed since the adherent cells that make up the microenvironment would not thrive in the vessels; (c) cytokine communications could be lessened by periodic changes of the medium; (d) stem cells, unlike differentiated cells, are self-renewing. Stem cells would outlast precursor cells. We consider the precursor cells those that have been committed to a specific differentiation.

Among approaches investigators have used in their quest for the hematopoietic stem cells in bone marrow are: density gradient centrifugation (Yoichi Takave, 1989), elutriation (Nijhof, et al., 1985), cell sorting by flow cytometry (McAlister, I., et al., 1990), immunomagnetic bead separation (Bertoncello, I., et al., 1989), and antibody-complement cytotoxicity (Spangrude, et al., 1988).

An efficient time and material saving protocol for stem cell isolation would help in gene therapy, bone marrow transplants, elucidation of cellular differentiation pathways, etc.

The work reported here includes a review of our work of the previous summer. We were confident that we had achieved the goal of stem cell isolation and characterization. We have since learned that the cells we described were infected with a virus and our conclusions became suspect. We have taken a number of different approaches to be used with the bioreactors including the use of Dexter medium with horse serum and hydrocortisone. We have also added some conditioned medium to the vessels. Our mice were certified pathogen-free.

MATERIALS AND METHODS

Mice: Certified pathogen-free CD2/F1 male mice 6-7 weeks old were purchased from Taconic Farms, Germantown, New York, 12526.

Cells: Mice were sacrificed by cervical dislocation. The bone marrow cells were obtained by flushing tibiae and femora of ten mice with Phosphate Buffered Saline (PBS) without calcium and magnesium and supplemented with 1% Bovine Serum Albumin. These cells were washed twice and resuspended in growth medium. The growth medium in one experiment was Dexter formulation: Iscove's Minimal Essential Medium (Sigma Chemical Co.) containing 20% horse serum (heat-inactivated at 56 deg C for 30 min) (HyClone, Upton, Utah) and 10^{-6} M hydrocortisone succinate sodium salt (Sigma). This medium was supplemented with 100 U/ml Penicillin-Streptomycin (Gibco, Grand Island, N.Y.). Cells in this medium were grown at 33 deg C.

In a second experiment the medium was that used in the summer, 1989: RPMI-1640 (Gibco), 10% Fetal Calf Serum (Flow Laboratories, Raockville, MD), and supplemented with Penicillin-Streptomycin (Gibco). The Fetal Calf Serum was heat inactivated at 56 deg C for 30 min. Cells in this medium were grown at 37 deg C.

Cell concentrations in both reactor vessels was 1×10^{-6} /ml. The vessel volumes were 50 ml. and were rotated at 8 rpm in an incubator in an atmosphere of 5% carbon-dioxide and 95% humidity. The bone marrow used in both controls and in experiments in microgravity initially was partially depleted of monocytes by removal of adherent cells by incubating the bone marrow cells overnight in polystyrene flasks at 37 deg C. in RPMI medium supplemented with 5% Fetal Calf Serum.

Simulated Microgravity: The cells were grown in one of NASA JSC's horizontally rotating culture vessels that simulates some aspects of microgravity. This vessel consists of a motor-driven rotating vessel with a separate air pump connected by tubing to the central shaft of the culture vessel. It operates in an ordinary laboratory tissue culture incubator. The cells are oxygenated by pumping 5% CO₂ to 95% incubator air through the internal silicone membrane which surrounds the central vessel shaft.

Cell counts. Cell counts were obtained on a standard Coulter Counter Model ZM (Coulter Electronics, Inc., Hialeah, FL, 33010) or by counting on a light microscope with a hemacytometer. Viabilities were determined by trypan blue exclusion.

Oxygen consumption and carbon-dioxide production were determined using a Corning 168, pH Blood Gas Analyzer (Corning, Medfield, MA, 02052). Glucose utilization was determined on a Beckman Glucose Analyzer 2 (Fullerton, CA, 92634).

Monoclonal Antibodies: The panel of antibodies included the following from clones purchased from American Type Culture Collection, Rockville, MD: TIB-104 (Lyt-1); TIB-120 (Anti Ia-b,d,q haplotypes); TIB-128 (Anti-MAC-1, macrophages and granulocytes); TIB-146 (Anti B cell, anti B cell precursors with antigen B220); TIB-150 (Anti Lyt 2.2 expressed on T suppressor-cytotoxic cells); TIB-1264 (Anti murine B cell); TIB-207 (anti-L3T4-T helper-inducer subsets). The following antibodies were from Sigma: FITC-anti Thy 1.2 (cell surface differentiation antigen), and the secondary antibodies FITC-anti Rat IgG and FITC Goat anti-mouse. We obtained from Becton-Dickinson Co., Mountain View, CA: Anti-mouse Lyt-1 and anti-mouse I-A. The anti-stem cell antigen antibody E13-161-7 was from ATACC. 2.4G2 (anti-mouse Fc receptor was gift of Dr. MaryAnn Principato of the National Institutes of Health, Bethesda, Md.

Staining: In direct staining, FITC-conjugated antibodies were used. In indirect staining, the unconjugated antibodies were incubated first with the bone marrow cells for 30 min on ice, washed, and incubated with the secondary FITC-conjugated antibody for 30 min on ice. After two washings, the cells were fixed for 10 minutes at 4 deg C with 3.7% formaldehyde, washed and resuspended in PBS-1% BSA/0.02% azide. If we had immediate access to the FACScan, the cells were not fixed but were incubated with a solution of propidium iodide which enabled us to sort out dead cells for the flow cytometric analysis.

Flow Cytometry. Experimental data from the control cell populations grown in static culture at unit gravity and from the cells grown in microgravity in the bioreactors were obtained using Coulter Electronics EPICS V Cell Sorter (Coulter Electronics, Hialeah, FL). 10,000 events were scored on each test. Dead cells and cell aggregates were gated from the cells under study using propidium iodide. The percentage of positive cells was estimated by using bone marrow with irrelevant antibody as controls. Cell size distribution was assayed by forward scatter of unseparated bone marrow cells. The percentage of cells with staining above the background is determined for each of the monoclonal antibodies in the panel. For DNA analysis, the cells were stained according to the Method of Krishan as modified by McDivitt, et al. Cells were fixed by suspension in a solution of 67% ethanol for 30 min. The staining solution was made of 0.005% propidium iodide (Calbiochem-Behring, San Diego, CA., 0.002 % RNase A (Sigma). After 30 min at 4 deg C the cells were washed and resuspended in PBS.

Stem Cell Bioassay. (Hemopoietic colonies in vitro). Stem cells in complete medium were added to soft agar formed colonies. The ratio of the colonies formed with the stem cell preparation and normal bone marrow cells was recorded. CFU-c was determined as described by Jacobs and Metcalf (1979). CFU-e was determined according to the method of McLeod (1974). BFU-e was determined as described by Peschle, et al (1979). CFU-GEMM was determined by the method of Johnson and Metcalf (1977).

Immunological Reestablishment; Three sets of ten mice each were irradiated with 900 rads at MD Anderson Cancer Center, Houston, TX. One set of the mice was kept as controls. A second set received fresh bone marrow injections. The test group was given 100 cell/mouse of the cells which had been circulating in the microgravity vessel for 40 days. Records were kept of animal viabilities.

Phenotypic and functional assays: (a) Among the surface antigens to be studied by our panel of antibodies are: Thy-1, CD3, CD4, CD5, CD8, H-2 Class I and II, slg, 14.8, SCA and Mac-1. (b) histochemical staining of the cells for monocyte detection will use specific esterase and for granulocyte/monocytes will use peroxidase.. (c) To determine if the cells secrete constitutively or after induction, we will use various hemopoietic growth and differentiation factors: IL-1, IL-2, IL-3 IL-4, CAF and INF-gamma. These phenotypic and functional assays were performed in the Laboratory of Dr. Chris Platsoucas of the M.D. Anderson Hospital in Houston, Texas.

RESULTS

Cells grown at unit gravity in T-flasks using Dexter formulation medium were our most successful cultures. They continued to produce non-adherent cells through a two month period.

The cells grown under the same conditions using RPMI-10% Fetal Calf Serum grew well for only a twelve day period. Cells in both Dexter medium and in RPMI-10%FCS medium in the bioreactor vessels declined in numbers steadily until there were very few viable cells by day 12. This was very unlike our three experiences in the summer of 1989. In the studies of last summer, the cell cultures were doubling every twenty-four hours by day twelve. Phenotypically they were the cells we sought -- the undifferentiated, hematopoietically pluripotent stem cells. We had apparently succeeded last year in producing a self-renewing immortalized cell line. Electron microscope photography indicated that the cells contained a virus. The virus could have accounted for their immortalization.

The cells grown with Dexter medium in the HARV did not survive beyond a few days; however similar cells grown in static culture in T-flasks at unit gravity were growing vigorously after five weeks. The cell suspension in the bioreactor by day twelve contained floating precipitate which we judged to be protein coming out of solution from the horse serum. The cells present had increased in size and resembled blasts. We concluded that the horse serum proteins precipitated which resulted in osmolarity and pH changes. Changes in either of these parameters would cause plasmolysis and lysis of the cells. We repeated the experiment several times and found the same results. We did not detect precipitated protein when the RPMI-10% Fetal Calf Serum was used; however, the cells did not survive after the 12 day incubation period. All of the horse serum in the Dexter composition was from a single batch; it is possible that this material was subject to denaturation in the vessels.

In the 1989 experiment we successfully monitored the conditions of the cultures in both static controls and in the rotating vessels using the parameters: osmolarity, pH, glucose and oxygen consumption and carbon dioxide production. We were also able to follow the depletion of the cells with lineage markers for cell surface antigens. The remaining, immortalized culture, was frozen away for future studies. When thawed a year later, the cells readily went into culture and continued to double in number daily. The cells stained positively for Thy-1 and for SCA-1. They were negative the Fc-receptor. The cells were negative to staining with the lineage markers.

The cells that were monitored for reestablishment of their immune systems in the 1989 experiments did not give us positive results. It may have been that the 900 rad radiation was too high a dosage; it may have been that we did not inject sufficient numbers of the stem cells from the rotating vessels. This test will be included in any study of stem cells isolated using microgravity.

Our conclusions are that the conditions that produce an environment of microgravity in the bioreactors will be most helpful after some basic science questions have been answered. There are apparently some growth factors needed from the stromal cell environment that are unique for the stem cell. This factor is different from a proliferation factor. There may be needed articulation with some stromal cells in the microenvironment formed by the adherent cell population. Our continued studies will consider each possibility.

DISCUSSION

This research was undertaken to determine the growth characteristics of murine bone marrow cells in the bioreactors of NASA-Johnson Space Center. Since these vessels offer an environment that partially simulates the microgravity environment, the data obtained would be relevant to the space program.

The major premises upon which this research was based are reasonable; if they are sound, we would expect the self-renewing stem cell to survive in the environment of microgravity and we would not expect differentiated cells to do so.

Our investigations have been concerned exclusively to this point with murine bone marrow cells. We want to expand the study to include human bone marrow. We are also anxious to explore the behavior of murine fetal liver cells in microgravity. The proliferative rates of fetal liver cells are considerably greater than bone marrow rates. They are also a self-renewing population.

We are confident that the microenvironment will yield the stem cell clone. The procedure must be "fine tuned" and optimal conditions and media identified. We would use cells from this clone to search for any antigens that might be unique to the stem cell. We would also seek pathways from the stem cell to terminally differentiated cells - myeloid, lymphoid, erythroid, as well as cells of current interest in medical research, e.g., the dendritic cell.

While we probably unintentionally immortalized the stem cell line in 1989, we are planning to transfect our isolated stem cells with a well defined oncogenic vector to produce an immortalized clone. In one experiment, canine bone marrow cells that were cultured for more than thirty weeks were found to be virally infected. (Schuening, F.G., et al, 1989). Murine bone marrow cells have been transformed in vitro with v-raf/v-myc retrovirus to yield mature B cells and macrophages. (Principato, M, et al., 1988) We would seek the stem cell clone using the same v-raf/v-myc vector. Retroviral vectors have been used with murine long-term bone marrow to transfect human glucocerebrosidase (Nolta, J.A., et al., 1990) and human adenosine deaminase (Wilson, J.M., et al, 1990). We are formulating protocols to transfect the stem cell clone if we succeed in isolating it. Erythropoietin cDNA has been transferred to murine bone marrow stromal cells and yielded hemoglobinized red blood cells. We would transfect our clone with cDNA and study the progeny. (Corey, C.A., et al 1989.)

The stem cell is deserving of further work in microgravity. Its isolation and characterization will be of particular relevance in bone marrow transplantation, immunodeficiency disorders, genetic engineering and long term space flights protection.

We are eager to study cells other than stem cells and self-renewing cells in microgravity, e.g., cells of the neuro-endocrine production.

The background preparation required for this study of murine bone marrow cells in simulated microgravity has been most rewarding. Our studies have prompted to ask many questions and now we must seek the answers to these questions.

Are bone marrow stem cells indefinitely self-renewing? They appear to be programmed for division. When one of them under stimulus is targeted to become a particular differentiated cell, then another stem cell seemingly undergoes mitosis. This appears to be the case since the percentage of stem cells seems to be the same in infants and in adult humans. The role of the stromal cell microenvironment in hematopoiesis is well documented. Specific stromal cell lines have been isolated and cloned and some their growth factors, differentiation factors and proliferative factors have been identified. Factors that inhibit the differentiation of stem cells have not been identified, if they exist. Neither have proliferation factors been identified that are specific for the stem cells.

It is important that the distinction between stem cells and precursor cells be defined. The literature is confusing. Investigators use the terms interchangeably. It is our conclusion that stem cells are programmed and once they are committed to become a specific differentiated cell line, they cease to be stem cells and become precursor cells. The reaction is apparently irreversible.

The bioreactor vessels which provide an environment of simulated microgravity is a major advance in cellular investigations. When the vessels become more widely available to investigators, reports of their applicability will appear in scientific publications. It is understandable that the efforts of the NASA engineers in providing such an apparatus will be a boon to the space program. The environment of space is one of hypogravity and conclusive evidence that some cells behave differently in altered gravity has been well documented. Humans in space will be exposed for longer periods in altered gravity as space exploration advances. The bioreactor vessels can serve as model systems for in vitro studies which could give information that could be extrapolated to situations that will be encountered on missions to Mars and beyond.

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SUMMARY

The data summarized graphically are from the successful culture growths of murine bone marrow in summer, 1989. The cells were grown in bioreactor vessels in an environment which simulated some aspects of microgravity.

Page 13-13. Test Run #1

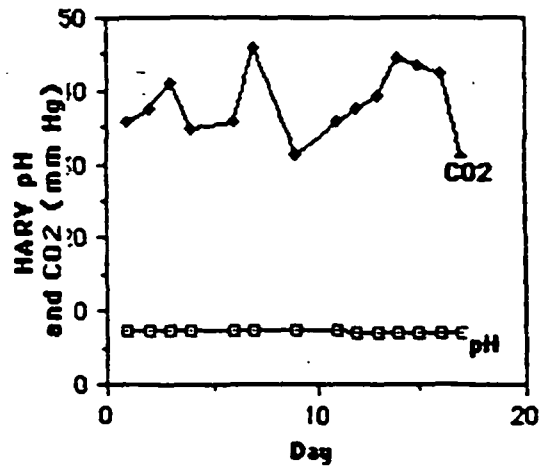
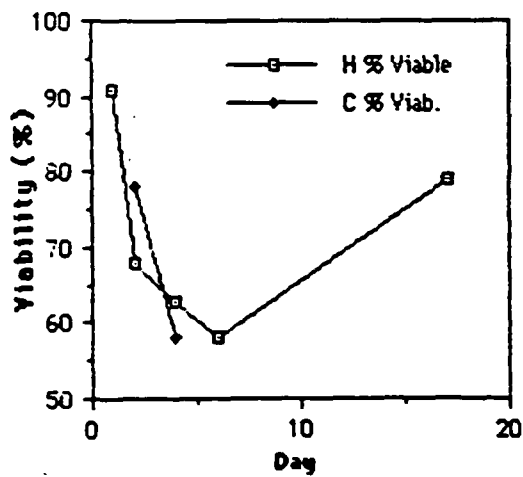
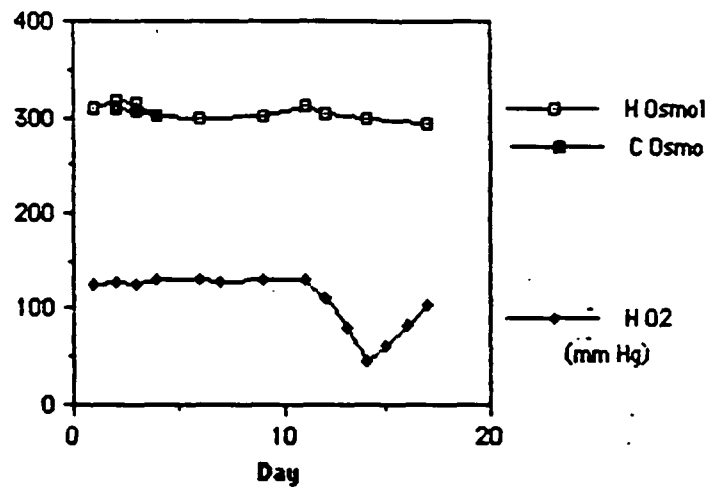
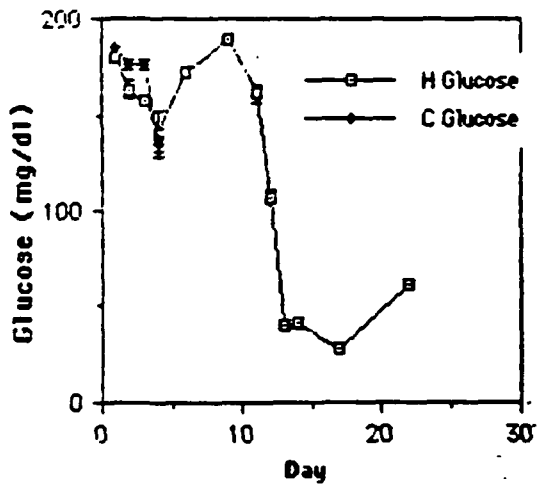
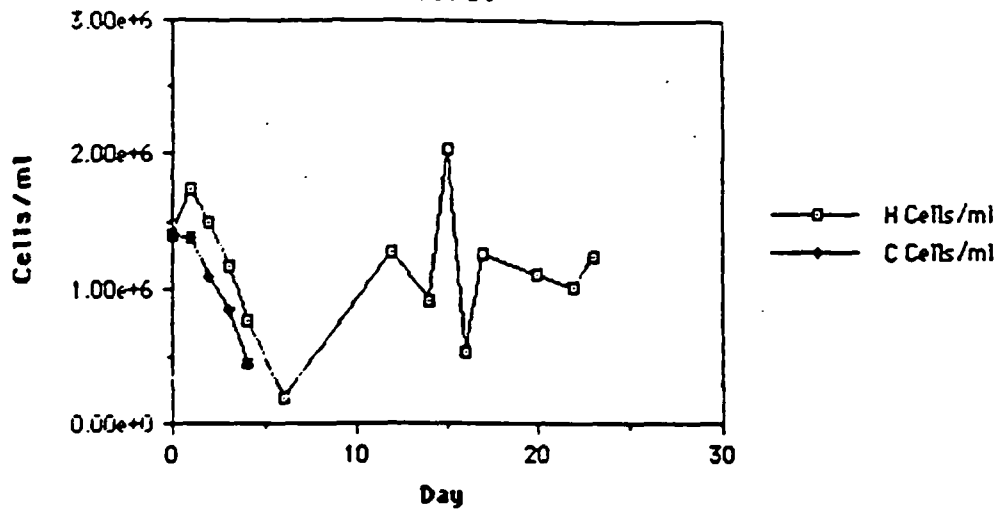
These cells were growing in simulated microgravity after 24 days. Their numbers at that time were doubling daily. The number of days of growth is plotted along the x axis and the log of the cell count number along the y axis.

Page 13-14 This graphical data is from the flow cytometer program. The cells monitored are shown to be positive for Thy-1 and negative for FcReceptor antigen. This data is consistent with that expected for stem cells. Fluorescence intensity is plotted on the abscissa and cell numbers on the ordinate.

Page 13-15. This data from flow cytometer shows that the cells monitored are positive for the "stem cell antigen" known as SCA-1. The clone which produces this antibody is available from ATCC. Fluorescence intensity is plotted against forward light scatter.

The program was gated to read only the viable cells in quadrant four.

6/19/89

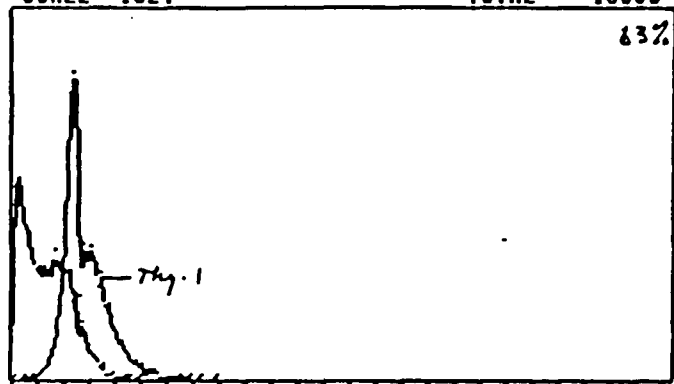


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SCALE= 1024

RESCALE

TOTAL= 10000
TOTAL= 10000

HIST 1
DOUBLE



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50 THY-1
LGFL /LRFL ,FALS

LAW 8/9 2:14 09AUG89.01500C
50 F CAR
LGFL /LRFL ,FALS

HALF

AUTO

HIST 2
DOUBLE

HALF

AUTO

LIN/
LOG

MERGE SCALE= 256
SCALE= 256

TWO-HISTOGRAM COMPARISON

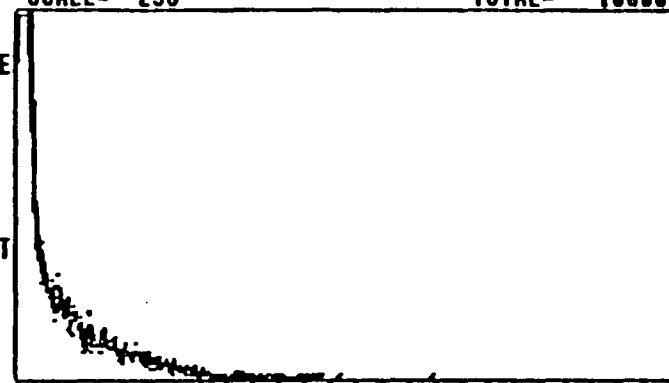
TOTAL= 10000
TOTAL= 10000

RESCALE

ADD

SUBTRACT

IMMUNO



KSTEST LAW 8/8 7:50 08AUG89.20700C

50 2462
LGFL /LRFL ,FALS

LAW 8/8 7:46 08AUG89.20500C

50 LYT-1
LGFL /LRFL ,FALS

FACS Analysis.

- Thy-1+ cells
- Lyt-1 and 2.4G2 (FcReceptor) -

QUADRANT STATISTICS

LAW 8/9 2:10 09AUG89.01300E
50 F GAR
FALS -LRFL

EXTENDED
ANALYSIS

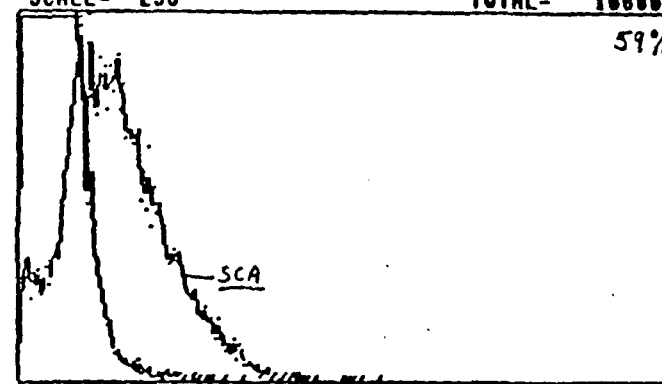
PRINT

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SCALE= 256

RESCALE

TOTAL= 10000
TOTAL= 10000

HIST 1
DOUBLE



HALF

AUTO

HIST 2
DOUBLE

HALF

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LGFL /LRFL ,FALS

AUTO

LAW 8/9 2:14 09AUG89.01500C
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LGFL /LRFL ,FALS

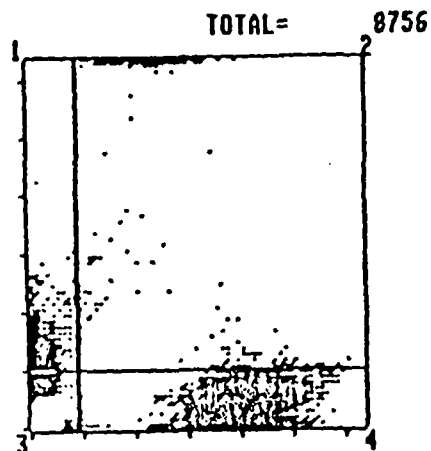
LIN/
LOG

RETURN

READY

RETURN

FALS 9
LRFL 10
LOWEST LEVEL
3



QUAD	PERCENT	PEAK POS	PEAK HT	AREA
1	10.88	0, 11	77	883
2	4.84	16, 63	10	354
3	26.30	0, 7	573	2303
4	59.57	30, 0	128	5216

READY, ADJUST CURSOR

FACS Analysis. Fluorescence Histograms. Fluorescence Intensity
on abscissa; cell numbers on ordinate.
a. Cell viability -- Cells in quadrant #4 analyzed.
b. Stem cell antigen+ cells